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Characterization of the Transcriptional *trans* Activator of Human Foamy Retrovirus

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The human foamy viruses, or spumaviruses, a distinct subfamily of complex human retroviruses, remain poorly understood both in terms of their pathogenic potential and in terms of the regulatory mechanisms that govern their replication. Here, we demonstrate that the human spumaretrovirus shares with other complex human retroviruses the property of encoding a transcriptional *trans* activator of the homologous viral long terminal repeat. This regulatory protein is encoded by the viral *Bel-1* open reading frame and is localized to the nucleus of expressing cells. The Bel-1 *trans* activator is shown to function effectively in cell lines derived from human, simian, murine, and avian sources. The viral target sequence for Bel-1 has been mapped 5′ to the start of viral transcription and is therefore likely to be recognized as a DNA sequence. Our results further suggest that the mechanism of action of the Bel-1 protein may be distinct from those reported for the transcriptional *trans* activators encoded by members of the other human retroviral subfamilies.

Humans are natural hosts to retroviral species belonging to each of the three subfamilies of the family *Retroviridae*. Both human T-cell leukemia virus type I (HTLV-I) and human immunodeficiency virus type 1 (HIV-1) have been shown to be the etiologic agents of specific human diseases (for a review, see reference 12). Infection with HTLV-I, a member of the oncovirus subfamily, can lead to severe neurological disease and is associated with the development of adult T-cell leukemia. HIV-1, a member of the lentivirus subfamily, is the causative agent of AIDS and is therefore a major human pathogen. In part because of their pathogenicity, these two viruses have been the subject of intense scientific scrutiny. These investigations have revealed that both HTLV-I and HIV-1 encode not only the structural gene products (Gag, Pol, and Env) characteristic of all replication-competent retroviruses but also several additional proteins that serve nonstructural or regulatory functions (7, 12, 26) (Fig. 1). In particular, both HTLV-I and HIV-1 have been shown to encode proteins that activate viral long terminal repeat (LTR)-dependent transcription (Tax in HTLV-I, Tat in HIV-1) or that modulate viral mRNA splicing (Rex in HTLV-I, Rev in HIV-1) (6–8, 12, 14, 15, 19, 26, 30, 31, 33).

The third subfamily of the *Retroviridae*, the foamy viruses, or spumaviruses, includes members able to replicate in a wide variety of species including cats, hamsters, cows, monkeys, chimpanzees, and humans (13, 35). These ubiquitous viruses are also able to grow in a range of cell types, including fibroblasts, and are relatively easy to isolate from their infected hosts (13). In fact, the human spumaretrovirus (HSRV) was the first human retrovirus to be cultured in vitro (1). In tissue culture cells, spumaviruses are highly cytopathic, giving rise to characteristic multinucleated syncytia that have a highly vacuolated, "foamy" appearance (13, 36). However, spumaviruses have yet to be clearly associated with any specific disease state and do not cause significant acute morbidity in their animal hosts (13, 36). In primates, spumaviruses appear to cause long-lasting, persistent infections marked by a high incidence of latently infected cells and by a wide tissue distribution, particularly including neural tissues (13, 35). In humans, HSRV infection has been tentatively associated with several pathogenic conditions, most particularly including de Quervain's thyroiditis (13, 36). However, the incidence of HSRV infection in the human population, and hence the relationship of HSRV infection to particular disease states, remains quite uncertain.

Because of the lack of a clear disease association, the human spumaviruses have until recently failed to attract the high level of scientific attention accorded the more clearly pathogenic human retroviral species. However, several subgenomic molecular clones of HSRV have been isolated and sequenced (9, 20). Recently, it has been shown that a full-length proviral clone of HSRV constructed from these subgenomic DNA fragments is fully infectious in tissue culture (17, 27). While this replication-competent HSRV proviral clone possesses the typical retroviral genomic organization, it also displays at least two interesting properties. The first is the exceptionally large size of the HSRV genome compared with other known retroviruses (Fig. 1). The second is the presence of at least three additional potential open reading frames (ORFs), termed *Bel-1*, *Bel-2*, and *Bel-3*, located between the *env* gene and the 3′ LTR (21) (Fig. 1). It has been proposed that these additional gene products could encode proteins functionally equivalent to the transcriptional and posttranscriptional regulatory proteins encoded by HTLV-I and HIV-1 (21). Recently, it has been demonstrated that cells infected by the human foamy virus, or the distantly related simian foamy virus, do indeed express a *trans* activator of foamy virus LTR-dependent gene expression (22, 28). In this article, we identify the *Bel-1* gene product as a nuclear *trans* activator of HSRV LTR-dependent transcription and present an initial characterization of the mechanism of action of this retroviral regulatory protein.

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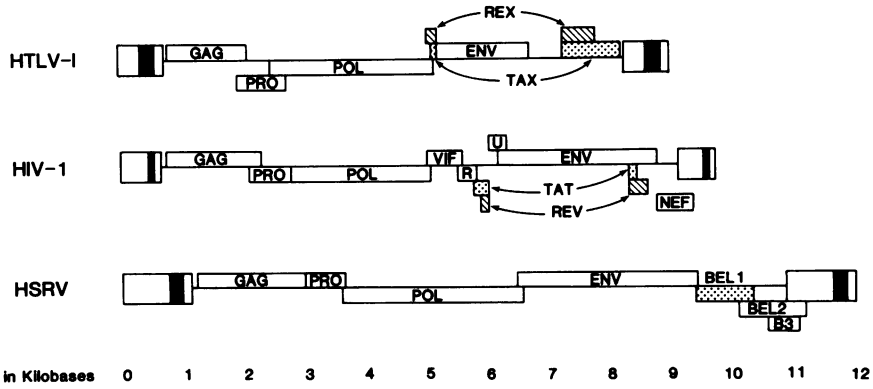

FIG. 1. Genomic organization of the prototypic human retroviruses HTLV-I, HIV-1, and HSRV. Viral genomes and known or proposed viral genes are named and drawn to approximate scale. LTRs are indicated by large terminal boxes with the repeated region solid. Viral transcriptional *trans* activators are indicated by stippled boxes, while known posttranscriptional regulators are indicated by hatched boxes. B3, Bel-3; R, Vpr; U, Vpu.

MATERIALS AND METHODS

Construction of molecular clones. All HSRV constructs were derived from the previously published pHSRV-B-C11, pHSRV-H-C55, and pHSRV-BS2 proviral clones (9, 20). Coordinates of HSRV sequences are given throughout relative to the site of viral transcription initiation. The overlapping *Bel* genes of HSRV were cloned by using the polymerase chain reaction (PCR) (23) and were expressed under the control of the cytomegalovirus (CMV) immediate early promoter present in the pBC12/CMV expression vector (4). PCR primers were designed to introduce a 5′ *Apa*I site and a 3′ *Ava*I site. The HSRV sequences present in each of the various Bel expression vectors were derived as follows: pBel-1+2+3, 8653 to 10405; pBel-1, 8653 to 9558; pBel-2+3, 9323 to 10405; and pBel-3, 9826 to 10319. Vectors encoding the HIV-1 *tat* gene (pcTat) and the HTLV-I *tax* gene (pcTax) have been described (19, 30).

We have previously described a vector, pBC12/HIV/SEAP, that contains the HIV-1 LTR linked in *cis* to the secreted alkaline phosphatase (SEAP) indicator gene (3). The pBC12/PL/SEAP vector was derived by replacing the HIV-1 LTR with a polylinker containing cleavage sites (ordered 5′ to 3′) for the restriction enzymes *Bgl*II, *Eco*RV, *Sal*I, *Hin*dIII, and *Sph*I. The pBC12/PL/SEAP vector is therefore suitable for the insertion of any promoter of interest immediately 5′ to the SEAP indicator gene. To generate pBC12/HTLV/SEAP, we excised the HTLV-I LTR from the previously described pU3R-I vector (33) by cleavage with *Xho*I (5′) and *Hin*dIII (3′). The HTLV-I LTR was then inserted between the *Sal*I (5′) and *Hin*dIII (3′) sites present in the polylinker of pBC12/PL/SEAP.

Nested deletion mutants of the HSRV LTR were generated by PCR with primers designed, in each case, to introduce a 5′ *Bgl*II site and a 3′ *Sph*I site. These DNA fragments were then inserted between the polylinker *Bgl*II and *Sph*I sites of pBC12/PL/SEAP. The full-length LTR construct, pBC12/HSRV/SEAP, contains viral sequences extending from the first base pair of the wild-type LTR 3′ unique (U3) region, at −777 relative to the cap site, to the initiation codon of the viral *gag* gene, at +446. Coordinates of the various deletion (pΔ) mutants of pBC12/HSRV/SEAP are listed in the text. A second indicator construction, termed pBC12/HSRV/CAT, is identical to pBC12/HSRV/SEAP except that the SEAP gene is replaced by the prokaryotic indicator gene for chloramphenicol acetyltransferase (CAT) (11).

Cell culture and DNA transfection. Cultures of the cell lines COS, QCl-3, L, and HeLa were maintained as previously described (4). HeLa cells were transfected by the calcium phosphate procedure, while COS, QCl-3, and L cells were transfected by using DEAE-dextran and chloroquine (5). SEAP enzyme assays were performed by using COS cell culture medium harvested at ~70 h posttransfection, as previously described (3). SEAP enzyme activity is given in arbitrary units derived from the rate of change in A_{405} measured in the standard SEAP assay. The levels of CAT enzyme activity expressed in transfected cultures were determined at 70 h posttransfection, as described by Neumann et al. (25).

Quantitative S1 nuclease protection assays were performed with total COS cell RNA harvested at 70 h posttransfection (4, 19). The probe strategy used to detect both SEAP mRNA and the RNA encoded by the internal control vector pBC12ΔI has been described (3, 4).

Immunofluorescence assays. A DNA fragment encoding amino acids 16 to 149 of the predicted 299-amino-acid *Bel-1* open reading frame fused to the N-terminal 98 amino acids of the polymerase protein of phage MS2 was expressed in *Escherichia coli* by using the prokaryotic expression vector pEX34 (18, 34). The Bel-1 fusion protein thus obtained was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18). A rabbit polyclonal antiserum raised against the recombinant Bel-1 protein (18) was shown to recognize authentic Bel-1 protein in HSRV-infected cells (17).

Indirect immunofluorescence assays were performed by using COS cell cultures transfected with pBel-1 or with a control vector, as previously described (5). The fixed transfected cells were treated with a 1:1,000 dilution of the primary rabbit anti-Bel-1 antiserum and a 1:50 dilution of the secondary antibody, a rhodamine-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim, Inc., Indianapolis, Ind.).

RESULTS

To test whether the HSRV proviral clone encoded a *trans* activator of the viral LTR, we positioned the HSRV LTR sequence 5′ to the SEAP indicator gene (3). This construct,

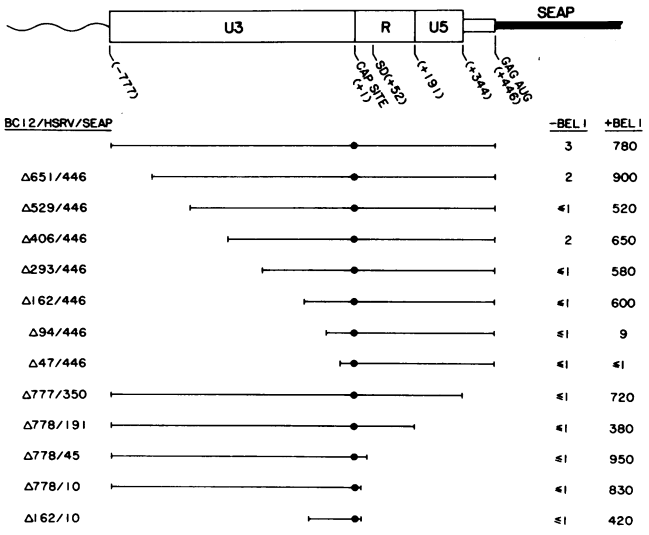

FIG. 2. Deletion analysis of the HSRV LTR. The pBC12/HSRV/SEAP vector contains the entire HSRV LTR, starting at the first base pair of the U3 region and extending through the repeated and U5 elements. In addition, pBC12/HSRV/SEAP contains viral leader sequences up to the *gag* translation initiation codon at +446, which has been changed to the initiation codon for the SEAP indicator gene product. Landmarks within the HSRV LTR, including the major viral splice donor (SD) sequence (24), are indicated and are given coordinates. Deletion (pΔ) mutants are named according to the extent of the remaining HSRV sequences; e.g., pΔ162/10 contains LTR sequences extending from −162 to +10. Levels of SEAP activity observed in cultures transfected with each HSRV LTR mutant in the presence and absence of Bel-1 were determined at 70 h posttransfection (3). The wavy line indicates flanking pBR322 sequences, while the dark circles indicate the viral mRNA cap sites.

termed pBC12/HSRV/SEAP, contained the entire HSRV LTR, starting precisely at the first base pair of the viral LTR U3 sequence and extending through the LTR repeated and U5 elements (Fig. 2). The SEAP indicator gene present in pBC12/HSRV/SEAP was positioned so that the translation initiation codon of SEAP precisely replaced the translation initiation codon of the viral Gag protein (Fig. 2). This construct therefore also contained all HSRV leader sequences normally located between the viral LTR and the *gag* gene.

The overlapping viral *Bel* genes were cloned into the eukaryotic expression vector pBC12/CMV by using PCR technology (23). The PCR primers used were designed to precisely excise specific ORFs, thus minimizing the presence of overlapping coding sequences (Fig. 1). The pBel-1+2+3 vector contained the entire *Bel* region starting immediately 5′ to the *Bel-1* ORF and ending 3′ to the *Bel-2* ORF. Similarly, pBel-1 contained all of *Bel-1* and a small part of the overlapping N terminus-encoding region of *Bel-2*. pBel-2+3 contained all of *Bel-2* and the fully overlapping *Bel-3* ORF, while pBel-3 contained all of the *Bel-3* ORF and the central part of the *Bel-2* ORF (Fig. 1).

The pBC12/HSRV/SEAP vector was transfected into COS cells together with each of the *Bel* expression constructs, and levels of SEAP activity in the supernatant media were assayed ~70 h later (3). The pBC12/HSRV/SEAP vector proved to have a very low basal activity that was at or below the sensitivity of the SEAP indicator gene assay (Table 1). However, cotransfection with the pBel-1+2+3 vector resulted in a dramatic, ~500-fold *trans* activation of the HSRV

TABLE 1. Bel-1 *trans* activation of HSRV LTR-dependent gene expression

Construct[a]	SEAP activity
pBC12/CMV	≤1
pBel-1+2+3	390
pBel-1	930
pBel-2+3	≤1
pBel-3	≤1

[a] Cultures were transfected (5) with equimolar amounts of pBC12/HSRV/SEAP together with each Bel expression construct or with the negative-control vector pBC12/CMV (4). Supernatant media were harvested at 70 h posttransfection, and levels of SEAP activity were determined as previously described (3).

LTR. A comparable level of *trans* activation was detected upon cotransfection with a *Bel-1* expression vector, termed pBel-1, that lacked most of the *Bel-2* and all of the *Bel-3* ORF (Table 1). In contrast, vectors predicted to express either the *Bel-2* or the *Bel-3* ORF did not induce detectable levels of HSRV LTR-dependent SEAP activity. We therefore conclude that *Bel-1* encodes a *trans* activator of HSRV LTR-dependent gene expression.

As noted above, both HTLV-I and HIV-1 have been shown to encode transcriptional *trans* activators of their homologous LTR promoters (6–8, 14, 15, 26, 31, 33). As an initial test of whether Bel-1 functioned via the same mechanism as either the HTLV-I Tax or the HIV-1 Tat protein, we next asked whether any of these viral regulatory proteins would cross *trans* activate the other human retroviral LTRs (Table 2). In fact, neither the Tax nor the Tat protein was found to exert any detectable effect on HSRV LTR-specific gene expression. Similarly, only the HTLV-I Tax protein was able to induce levels of HTLV-I LTR-dependent gene expression that were detectable in this assay system (Table 2). In the case of HIV-1, a readily detectable basal level of LTR-dependent gene expression was detected, and this was increased by ~40-fold in the presence of the Tat *trans* activator. As previously shown by others (32), the HTLV-I Tax protein acts as a moderate *trans* activator (~10-fold) of the HIV-1 LTR. Of interest, the Bel-1 protein also appears

TABLE 2. Bel-1 *trans* activator specificity for the HSRV LTR

Clones transfected[a]	SEAP activity
pBC12/HSRV/SEAP plus:	
pBC12/CMV	≤1
pBel-1	750
pcTax	≤1
pcTat	≤1
pBC12/HTLV/SEAP plus:	
pBC12/CMV	≤1
pBel-1	≤1
pcTax	10
pcTat	≤1
pBC12/HIV/SEAP plus:	
pBC12/CMV	16
pBel-1	130
pcTax	170
pcTat	600

[a] Assays were performed as described in the footnote to Table 1.

TABLE 3. Bel-1 activity in a range of cell lines

Plasmid	CAT activity[a] in cell line			
	COS	HeLa	L	QCl-3
pBC12/HSRV/CAT + pBel-1	42,590	19,230	20,280	66,820
pBC12/HSRV/CAT + pBC12/CMV	220	230	200	100
pBC12/CMV[b]	210	210	170	160

[a] The relative level of CAT activity detected in each culture is given in counts per minute, as determined by the method of Neumann et al. (25). A similar level of cell extract was assayed for each cell line tested, except COS, for which one-fifth of the level of extract was used. Because cell transfectability varies significantly, the absolute level of CAT expression cannot be meaningfully compared between different cell lines.

[b] Negative control (culture transfected with the parental plasmid only).

able to moderately (~8-fold) activate HIV-1 LTR-dependent gene expression.

Foamy retroviruses are ubiquitous viruses with an unusually wide species distribution (13, 35). We therefore wished to examine whether Bel-1 would be able to activate HSRV LTR-specific gene expression in nonprimate cells. In order to enhance the sensitivity of this analysis, we used a construct, pBC12/HSRV/CAT, in which the HSRV LTR was linked in *cis* to the prokaryotic CAT indicator gene (11). In addition to the simian COS cell line, the cell lines tested were of human (HeLa), mouse (L cells), or quail (QCl-3) origin. In each case, cells were transfected with the pBC12/HSRV/CAT plasmid in the presence or absence of the Bel-1 *trans* activator (Table 3). At ~70 h after transfection, cultures were lysed, and CAT expression levels were determined (25). In each cell line, the level of HSRV LTR-specific CAT expression was observed to be dramatically enhanced in the presence of Bel-1 (Table 3). CAT expression in the absence of Bel-1 was essentially indistinguishable from background levels, thus precluding any reliable estimate of the absolute level of *trans* activation.

We next attempted to map the sequences within the large HSRV LTR that were responsive to the Bel-1 protein. In particular, we wished to determine whether the target sequence for transcriptional *trans* activation was located 5′ to the cap site, as observed in the case of HTLV-I (15, 31), or was located 3′ to the cap site, as observed for HIV-1 (6). For this purpose, we derived a nested set of 5′ and 3′ LTR deletion (pΔ) mutants from the parental pBC12/HSRV/SEAP vector (Fig. 2). Each deletion mutant was named according to the most 5′ and most 3′ HSRV base pairs remaining in the vector. As shown in Fig. 2, deletion of LTR U3 sequences between −777 and −162 had no significant effect on HSRV-dependent gene expression in the presence of the Bel-1 *trans* activator. However, further deletion of U3 sequences between −162 and −94 in pΔ94/446 resulted in a marked attenuation in the level of HSRV gene expression in the presence of Bel-1. A further deletion of U3 sequences, between −94 and −47 relative to the cap site, prevented detectable HSRV LTR-dependent SEAP gene expression even in the presence of Bel-1. In contrast, a set of 3′ deletions that extended to within 10 bp of the HSRV LTR transcription initiation site had little or no effect on the responsiveness of the HSRV LTR to the *Bel-1* gene product (Fig. 2). A final HSRV LTR deletion mutant, termed pΔ162/10, was constructed to demonstrate that sequences located outside of this minimally responsive LTR sequence were indeed dispensable for *trans* activation by the Bel-1 protein.

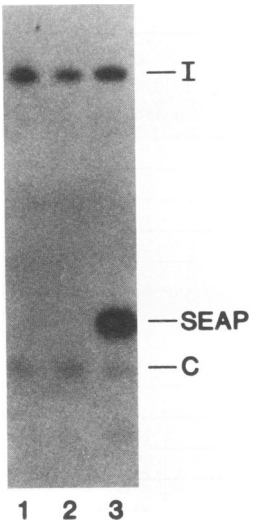

FIG. 3. Bel-1 activates HSRV LTR-dependent RNA expression. COS cell cultures were transfected with 5 μg of pBel-1 (lane 1), with 5 μg of pBC12/HSRV/SEAP (lane 2), or with 2.5 μg of each (lane 3). In addition, each culture was cotransfected with 50 ng of the Rous sarcoma virus LTR-based internal control construction pBC12ΔI (4). The Rous sarcoma virus LTR is not responsive to the Bel-1 *trans* activator. Total cellular RNA was harvested at 70 h posttransfection (19) and subjected to quantitative S1 nuclease analysis by using an input probe (I) able to detect both SEAP mRNA (SEAP) and the internal control RNA (C) (3, 4). HSRV LTR-derived SEAP RNA was detected only in the presence of the Bel-1 *trans* activator (lane 3).

On the basis of these observations, we suggest that the target sequence for Bel-1 is located within the HSRV LTR U3 region and is likely to extend 5′ to the −94 position relative to the viral LTR cap site.

The localization of the LTR target sequence for Bel-1 5′ to the start of viral transcription strongly suggested that Bel-1 activated HSRV gene expression via a transcriptional mechanism. To further examine this possibility, we assayed the level of HSRV LTR-dependent SEAP RNA expression in transfected COS cells in the presence and absence of the Bel-1 *trans* activator (Fig. 3). No SEAP RNA was detected in the absence of Bel-1 (Fig. 3, lane 2), while the indicator RNA became very readily detectable in the presence of this viral *trans* activator (Fig. 3, lane 3). We therefore conclude that Bel-1 is the transcriptional *trans* activator of HSRV LTR-dependent gene expression that has been reported in foamy virus-infected cells (22, 28).

In a final experiment, we addressed the subcellular localization of the HSRV Bel-1 protein. For this purpose, we raised a rabbit antiserum directed against a bacterially synthesized Bel-1 fusion protein. This rabbit antiserum was then used for indirect immunofluorescence analysis (5) of fixed COS cell cultures that had been transfected with either pBel-1 or a control vector. This experiment (Fig. 4) revealed that Bel-1 is localized exclusively to the nucleus of expressing cells but is excluded from the nucleolar region. This localization is therefore similar to that observed for the HTLV-I Tax protein (8, 10), as well as for other viral DNA sequence-specific *trans* activators, such as the simian virus 40 T antigen and the polyomavirus large T antigen (16, 29). However, this subcellular localization is distinct from that observed for the RNA sequence-specific HIV-1 Tat *trans*

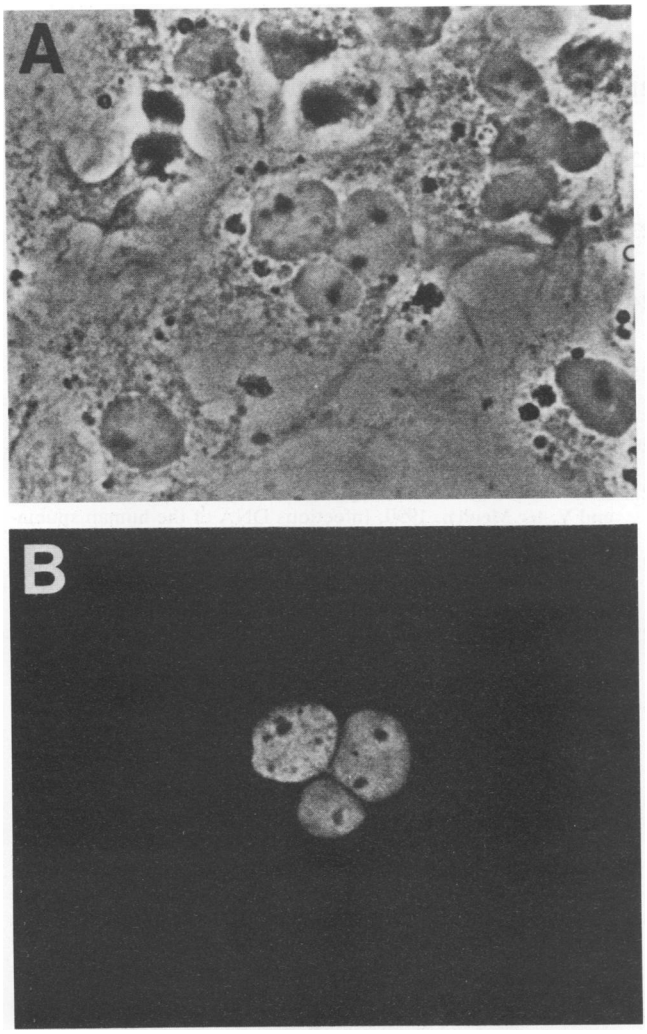

FIG. 4. Subcellular localization of the HSRV Bel-1 protein. Phase-contrast (A) and corresponding immunofluorescence (B) photographs of COS cells transfected with pBel-1. Cells were fixed, permeabilized, and stained at 70 h after transfection (5) with a rabbit polyclonal anti-Bel-1 antiserum. Nuclear expression of Bel-1 was detected in ~5% of the transfected cells, the percentage expected to have incorporated plasmid DNA as the result of our transfection procedure (5). No positive cells were detected in cultures transfected with a negative control vector (data not shown). Magnification, ×266.

activator, which is known to concentrate in the nucleoli of expressing cells (6).

DISCUSSION

In this article, we describe an initial investigation of the regulatory events that govern gene expression in the human foamy viruses. We confirm that the HSRV provirus encodes a highly effective *trans* activator of HSRV LTR-dependent transcription (28) and identify this regulatory protein as the product of the viral *Bel-1* gene (Table 1). The Bel-1 *trans* activator was observed to be active in cells drawn from a wide range of eukaryotic species (Table 3), thus suggesting that any cellular cofactor(s) required for Bel-1 function must also be widely distributed. Although the basal activity of the HSRV LTR was barely detectable in our assay systems, the Bel-1 *trans*-activated LTR was observed to give a level of gene expression comparable to those of the most active promoters available in this laboratory, including the CMV immediate early promoter, the Rous sarcoma virus LTR, and the Tat *trans*-activated HIV-1 LTR (Table 2 and data not shown). The target for the Bel-1 *trans* activator was shown to be located 5′ to the HSRV transcription start site, within the viral LTR U3 region, and is therefore recognized as a DNA sequence. As predicted for a DNA sequence-specific transcriptional activator (8, 16, 29), Bel-1 was found to localize to the cell nucleus but to be excluded from the nucleolus. It is therefore evident that HSRV shares with other complex human retroviruses the property of encoding an activator of viral LTR-dependent gene expression.

The Bel-1 *trans* activator is similar to the HTLV-I Tax protein, and distinct from the HIV-1 Tat protein, in that the target for *trans* activation appears to be a DNA, rather than an RNA, sequence (6, 15, 31). However, the Bel-1 protein lacks the ability to activate HTLV-I LTR-specific gene expression, and the HTLV-I Tax protein is similarly unable to enhance transcription from the HSRV LTR (Table 2). In addition, there are no sequences within the HSRV LTR that display significant identity with the known viral Tax target sequence, the HTLV-I LTR 21-bp-repeat element (15, 31) (data not shown). The mechanisms of action of Tax and Bel-1 therefore appear likely to be distinct. However, it is noteworthy that both Tax and Bel-1 do moderately *trans* activate the HIV-1 LTR. In the case of Tax, it has been proposed that this activation is due to the induction of the cellular transcription factor NF-κB (2). It will therefore be of interest to determine whether Bel-1 activates the HIV-1 LTR by a similar mechanism.

Deletion mutagenesis of the HSRV LTR has shown that sequences located between −162 and −94 relative to the HSRV cap site are essential for full *trans* activation by Bel-1 (Fig. 2). Although we cannot exclude the possibility that these sequences are basal promoter elements required only indirectly for Bel-1 function, we favor the alternate hypothesis that the target for Bel-1 extends 5′ to −94 in the HSRV LTR U3 region. Inspection of this region has failed to reveal consensus sequences for any of several established eukaryotic transcription factors, including NF-κB, AP-1, and CRE. It will therefore be of interest to define the precise target sequence for Bel-1 and to establish whether this potent *trans* activator is indeed acting via a novel activation pathway.

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